Production and characterization of chitosan nanoparticles extracted from Pinus roxburghii needles enhances disease protection against citrus canker

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Research Article

Keywords: Encapsulation, Antibacterial activity, Chitosan, Pinus roxburghii, Zinc oxide nanoparticles, Microwave-assisted encapsulation, Lyophilization

Posted Date: August 12th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-7226794/v1

Abstract

During recent years, nanotechnology has emerged as a promising technology to combat plant diseases.. This present research aimed to synthesize chitosan coated zinc oxide nanoparticles from *Pinus roxburghii* needles to mitigate the canker disease. Freeze drying and microwave-assisted methods were applied for encapsulation of synthesized nanoparticles. For characterization of nanoparticles UV-Vis, SEM, XRD, FTIR, DLZ, and zeta potential were employed. Results of the present work indicated that the synthesis of nanoparticles has hexagonal Wurtzite structure, while SEM depicted morphology and size. In contrast to the amorphous needles extract and chitosan, the crystalline nature of the nanoparticles is demonstrated by the strong crystalline peaks of XRD between 20 < 2Θ >80° before and after encapsulation. Particle size of 68 nm was determined by DLS that had surface charge of -25.66 mV as determined by zeta potential. Antibacterial activity of ZnO-NPs of *Pinus roxburghii* extracts with and without encapsulation was evaluated against pathogenic bacteria *Xanthomonas citri*. Higher antibacterial activity is exhibited by nanoparticles encapsulated through the microwave-assisted method as compared to freeze drying. Antimicrobial activity of needle extract increased by increasing the concentration of chitosan-coated zinc oxide nanoparticles. In conclusion, nanoparticles of *Pinus roxburghii* have antibacterial effects against *Xanthomonas citri*, and their activity increases due to encapsulated chitosan for citrus canker disease

1. Introduction

As the global population continues to grow, there is an increasing concern over food availability and food production. On the other hand, the decline in crop quality and output due to plant disease is a persistent threat to global food security, as most of our food comes from plants [1]. The citrus industry, in particular, suffers substantial losses from several diseases, including citrus canker, leading to reduction in fruit quality [2, 3]. Besides citrus industries other industries that depend on citrus plants such as food processing industry, beverage industry, cosmetics and personal care industry, pharmaceutical and nutraceutical industry, cleaning and chemical industry, agriculture and animal feed, rely on the cultivation, harvesting, processing, and chemical extraction of various components from citrus plants, highlighting their significant economic importance beyond just the fresh fruit market [4–7]. Symptoms include fruit defects, defoliation, twig dieback, premature fruit drop, and tree decline, all resulting from erumpent lesions on stems and leaves caused by pathogens [8]. Three major types of citrus canker disease (A, B, and C) are caused by *Xanthomonas* Gram-negative bacteria [9]. Various strategies are employed to control citrus canker, including using resistant cultivars, eradicating infected plants, pruning, burning, and applying fungicides and copper sprays [10]. However, these methods can be costly, labor-intensive, and potentially toxic to animals, humans, and beneficial microorganisms [8–11].

Plant extracts and other essential oils are used as antimicrobial agents because of their bioactive compounds and higher antioxidant potential, as well as being eco-friendly [11]. Oil extracted from *P. roxburghii* is rich in bioactive compounds, including flavonoids and phenolics, and has notable medicinal therapeutic. and antimicrobial values[12]. In addition, bioactive compounds, such as α-terpineol, α-

humulene, α-thujene, L-bornyle-acetate, 4-terpineol, Trans-pinocarveol, terpenolene, δ-cadinene, α-carryophyllene, β-caryophyllene, α-terpinol limonene, camphene, L-β- pinene, β- pinene, carryophyllene, 3-carene, and α-pinene were identified in the pine needles [7, 13, 16–18].

Nanoparticles have emerged as a promising approach for addressing plant diseases due to their crystalline structure and high structure-to-volume ratio, contributing to enhanced chemical activity. Researchers have demonstrated that nanoparticles can be effective antibacterial agents [16–18]. Various chemical and physical techniques are utilized to produce nanoparticles. The biological approach, called green synthesis, has gained significant prominence and attracted considerable attention due to its cost-effectiveness, environmentally benign nature, and non-toxic characteristics. In this process, metallic ions from their compounds (e.g., zinc ions from the zinc acetate) react with organic compounds in the plant extract, which act as reducing agents, leading to the formation and stabilization of nanoparticles (Fig. 1).

The potential of phytochemical-rich pine plant materials in nanoparticle synthesis has gained considerable attention in recent years [25]. For instance, different nanoparticles were produced using needles, bark, and cones of *P. eldarica* and *P. merkusii*[26, 27]. Gold NPs were derived from the pollen of *P. kesiya*[28], and silver NPs were extracted from the stem of *P. wallichiana* [29]. Nanoparticles produced from silver using needles of *P. densiflora* exhibited antibacterial activity against both gram-positive and gram-negative bacteria, offering plant disease resistance and production of biomediated nano-pesticide [19]. The higher accumulation of phytochemicals in different parts of *P. roxburghii* makes it a potential source of nanoparticle production and plant disease management [20, 21].. The higher accumulation of phytochemicals in different parts of *P. roxburghii* makes it a potential source of nanoparticle production and plant disease management [31, 32]. However, it is still unknown whether *P. roxburghii* needle extracts can synthesize nanoparticles that can fight plant pathogens [22]. Exploring the potential of *P. roxburghii* in this context could provide new avenues for sustainable plant disease management strategies.

Nanotechnology offers promising solutions for enhancing the delivery and efficacy of plant-based treatments. Nanoparticles act as carriers for transporting plant extracts, augmenting stability and facilitating permeability across the cell membranes (see Fig. 2) [23]. A range of materials, such as polymeric nanoparticles, has been utilized for plant extract transportation [24, 25]. These nanoparticles have the capacity to encapsulate extracts or other significant components within the nanocarrier, owing to their minimal toxicity to biological systems, remarkable stability, exceptional biocompatibility, biodegradability, and ability to enhance antimicrobial efficacy [26–28]. The extract-containing nanoparticles are further encapsulated to enhance their antimicrobial activity [29, 30] and stability through various encapsulating methods [31, 32]. This approach represents a significant advancement in plant disease management, offering a sustainable and effective method of protecting crops.

Nanoencapsulation, which involves trapping bioactive compounds within nanoparticles, enhances the stability, solubility, and bioavailability of these compounds while protecting them from adverse

conditions such as heat degradation [33]. The present study proposes a novel and sustainable approach to synthesizing ZnO-NPs using extracts from *P. roxburghii* needles for plant disease management. Utilizing microwave-assisted and freeze-drying procedures for encapsulating nanoparticles via emulsion techniques is a valid approach [34, 35]. A range of synthetic and natural polymers is employed, with the desirable characteristics of biodegradability, biocompatibility, and safety. Chitosan, known for its biocompatibility, biodegradability, and natural antibacterial potential, is proposed for this study. These properties of chitosan make it a suitable choice for encapsulation of nanoparticles. The encapsulated nanoparticles may assist in slow release and provide long-term protection against plant pathogens and diseases [36, 37].

Bionanomaterials involving ZnO-NPs typically interact directly with bacterial membranes and penetrate the molecular structure, such as DNA and RNA, inhibiting bacterial growth and eventually destroying cellular outputs. In addition, ZnO-NPs generate positive ions of zinc ions, inhibiting enzymatic activity and, eventually, causing bacterial cell death. The study aimed to assess the impact of synthesized zinc oxide nanoparticles from *P. roxburghii* needle extract for controlling citrus canker disease. Additionally, the study aimed to evaluate the antimicrobial efficacy of both the extracts and nanoparticles, both prior to and subsequent to encapsulation, against plant pathogens.

2. Materials and Methods

2.1. Synthesis of Zinc oxide nanoparticles

2.1.1. Extract preparation

Pinus roxburghii extract was prepared by following the procedures utilized in previous study [46, 47]. A quantity of 2–4 g of *P. roxburghii* needles powder was added to 120 mL of ethanol/distilled water solvent (59.6: 40.4 or 60:40). The flasks were placed in a water bath and maintained at a temperature of 60°C for a duration of 30 minutes. The final extracts of the pine needles were obtained by filtering the solutions using Whatman filter paper. The pH of the extract solutions was determined using a pH meter, yielding a value of 6.97.

2.1.2. Nanoparticles synthesis

ZnO-NPs were prepared by using plant extracts (120 mL), placed in a magnetic stirrer without heat at 900 rpm. Ten (10 mM) zinc acetate salt was prepared by dissolving zinc acetate with the 1:2 incluiding pine powder in 60 mL of distilled water [48]. The plant extracts were mixed with salt dropwise using a micropipette at 5-minutes intervals for 2.5-3 hours. The synthesis involves controlled precipitation of zinc oxide from zinc acetate precursors under mild heating and stirring, without any change in oxidation state. To dissolve the salt, the flasks were vigorously shaken overnight at 37°C and 120 rpm in a shaking incubator. Absorbance was measured using a spectrophotometer. The pH level of the nanoparticle solutions was maintained at 6.3 by using NaOH and HCl to adjust the pH as necessary. The nanoparticle solution was centrifuged for 20 minutes at 6000 rpm and 27°C. The supernatant was discarded, and the

pellet was washed thrice with distilled water to remove impurities. The pellet was oven-dried at 60°C for about 2 hours. The nanoparticles were calcined at 300°C for 1–3 hours in a Muffle furnace to fix the active ingredients or compounds.

2.2. Optimization of zinc oxide nanoparticle synthesis

Response surface methodology (RSM) was used to optimize the conditions for synthesizing ZnO-NPs from *P. roxburghii*, which simultaneously exhibits the interaction between various factors at different levels. Design Expert software version 11.0 was employed to evaluate and design the experimental design. The central composite design (CCD) of three variables was used to design the response surface model [49, 50]. A model was contracted to reduce the total number of runs (experiments), and twenty-five experiments were run for the nanoparticle synthesis from the pine plant. Three independent variables, pH (4.2-7), temperature (20–37°C), and salt concentration (0.6–2.3 g), were considered in an experiment to evaluate their impact on the response variable (absorbance). UV absorbance was selected as the response variable because it provides a rapid, non-destructive indication of nanoparticle formation and concentration, and is widely used in plant-mediated nanoparticle synthesis studies to monitor nucleation kinetics. Design Expert software version 11.0 was used to generate the model and analyze interactions among variables.

2.3. Encapsulation of zinc oxide nanoparticles in chitosan (by using microwave-assisted method)

The aqueous solution of the chitosan was formed by adding 0.5 g of chitosan (1 M) with 20 mL double-distilled water at 50 ± 1°C and 1% acetic acid and mixing it thoroughly with a magnetic stirrer. The nanoparticle solution of 5 ml, with the help of a micropipette, was added dropwise into the beaker containing an aqueous chitosan solution. A ratio (1:1) of the guest molecule (synthesized ZnO-NPs of pine needles extracts) with the host molecule (aqueous solution of chitosan) was mixed for an hour continuously by using a magnetic stirrer at 200 rpm. After one hour, the speed increased to 1000 rpm in 15 minutes. Using the microwave-assisted encapsulation method [51], the mixture was placed in 5 mL porcelain crucibles and heated with a maximum incident microwave power of 200 W for 30 min with the ON/OFF cycle of the microwave for every 30 sec. The temperature at 80℃ was maintained for the encapsulation of ZnO-NPs in the chitosan matrix. The obtained encapsulated powder was grounded for 5 minutes using a mortar and pestle to ensure breakage of the lumps formed during the encapsulation process, and the obtained powder was uniform. The resulting powder was ground and stored at -20°C, and glassware was meticulously cleaned to prevent contamination. The encapsulation results were evaluated through UV-Vis, FTIR, XRD, and SEM analyses.

2.4. Encapsulation through Lyophilization (or freeze-dry method)

2.4.1. Extraction of pine needle extracts

Fifty grams of powdered pine needles were combined with 250 mL of an ethanol/water solvent (59.6 40.4 or 60:40). The solution was then filtered using Whatman filter paper and heated in a water bath (or ultrasonic bath at 35 KHz frequency) for 50 minutes at 65°C. Pellets were collected in a non-sticky container after being centrifuged at 3500 rpm for 10 minutes to separate the extracts. The pellet was baked at 40°C for 5 to 8 hours, dried, and then ground into a powder for long-term storage at room temperature.

2.4.2. Preparation of biopolymer solution

A solution was prepared by adding 0.5% locust bean gum to deionized water, which was then subjected to magnetic stirring at a temperature of 20℃ for 15 minutes. The mixture underwent refrigeration for approximately 24 hours. A second solution containing 2% acetic acid was supplemented with 0.5% chitosan in a volume of 1000 ml and underwent agitation for 30 minutes at 20℃. The formation of a complex solution between locust bean gum and chitosan was achieved by combining the two solutions and subjecting them to stirring at a temperature of 20℃.

2.4.3. Preparation of water-in-oil and oil-in-water double emulsion

2.4.3.1. First water in oil micro-emulsion

In the continuous phase, 25% of Tween 20 and 68% soybean oil, 7% of plant extracts were added dropwise to make the first emulsion.

2.4.3.2. Second, coating of water in oil micro-emulsion with biopolymer

In the second emulsion, 30% of the initial water in the oil emulsion was added to 70% prepared biopolymers. The solution was then homogenized at 12,000 rpm for 5 minutes at 10℃ at a magnetic stirrer. After that, the speed of homogenization was increased to 18,000 rpm for 8 minutes and then freeze-dried for up to 48 hours[52].

2.5. Characterization of nanoparticles

The synthesis of ZnO-NPs was confirmed through color change, UV-Vis spectroscopy, XRD, FTIR, DLS, zeta potential and SEM analysis. **2.6 Antimicrobial activity of pine extracts and nanoparticles before and after encapsulation**

2.6.1. Disc Diffusion Method

Media was prepared by dissolving 2.8 g of nutritional agar in 100 mL of distilled water. The sterilized, autoclaved media were poured into the sterile petri plates and solidified at room temperature in a laminar flow cabinet. Liquid media was made by adding 0.26 g of nutrient broth in 20 mL of distilled water and then autoclaving. The inoculum was made by adding *X. citri* bacteria with a sterilized loop to

the liquid media and placing it in a shaking incubator at 37°C for 24 hours. After 24 hours, the inoculum of the bacteria was spread on the petri plates. Pine oil, pine needle extracts (both encapsulated and non-encapsulated), and ZnO-NPs of pine needle extracts (both encapsulated and non-encapsulated) were tested, along with antibiotic and water as positive and negative controls, on nutrient agar petri plates inoculated with a bacterial culture. Plates were incubated at 37°C for 24 hours. After 24 and 48 hours, the bacterial zone of inhibition was evaluated.

2.6.2. Minimum Bactericidal and Minimum Inhibitory Concentration

2.6.2.1. Broth dilution

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antibacterial agent that may prevent bacterial growth. Test tubes containing 5 mL of Nutrient Broth and ZnO-NPs at final concentrations of 0.05, 0.5, 1.0, and 2.0 mg mL^{-1} were used for quantitative evaluations. Nutrient broth without ZnO-NPs served as a control. The test tubes were loaded with the working culture to reach an initial cell concentration of about 105 CFU mL^{-1}, then incubated at 37°C with shaking. Cell concentrations were determined after 0, 24, and 48 hours of incubation by serial dilution in distilled water and spread plating on Plate Count Agar (PCA) with some modification as previously described by [55].

2.6.2.1. Growth curve of microbial strain

The bacterial strain's growth curve was studied in a culture medium containing ZnO-NP suspensions at their respective MIC and MBC, as described in [55].

The MBC for the microorganism was determined using the dilution in broth method[56]. Volumes of 1 and 2 mg mL^{-1} of ZnO-NPs were inoculated on nutritional agar plates for MBC, the plates were incubated at 37°C for 24, 48, and 72 hours,

3. Results and discussion

In this study, we aimed to optimize the synthesis of zinc oxide nanoparticles (ZnO-NPs) using *Pinus roxburghii* needle extracts and to evaluate their antimicrobial efficacy against citrus canker disease. Various characterization techniques were employed to confirm the successful synthesis and encapsulation of the nanoparticles. The results highlight the potential of using plant-derived nanoparticles as an eco-friendly alternative for plant disease management.

3.1. Optimization of nanoparticles through CCD

The current study evaluated the correlation between various independent variables, namely pH, process temperature, and salt concentrations, in producing green synthesized chitosan ZnO nanoparticles

utilizing extracts from pine needles. The Central Composite Design (CCD) was employed to optimize the synthesis conditions of zinc oxide nanoparticles (ZnO-NPs) using *P. roxburghii* needle extracts. The CCD is a robust statistical tool used to evaluate the effects of multiple factors and their interactions on response variables, enabling the determination of optimal conditions for desired outcomes[49, 50].

Based on the analysis of variance (ANOVA), the factors of salt concentration ($p = 0.0249$), temperature ($p = 0.0033$), and pH ($p < 0.0001$) exhibited statistical significance in relation to the absorbance (response variable) ($p < 0.05$). UV absorbance was used as a surrogate indicator of nanoparticle formation because it offers a rapid, non-destructive, and reproducible signal correlated with ZnO crystal nucleation. It is commonly applied in plant-based synthesis systems to track real-time nanoparticle development and optimize reaction efficiency. The pH variable exhibited the highest F-ratio, around 46.38, and the lowest *p* value (< 0.0001). Based on these measurements, the pH of the solution showed the most significant impact on the UV absorbance of the nanoparticles. Secondly, a temperature *p* value of around 0.0033 and an F-ratio of 12.19 was recorded, followed by the salt, which showed the lowest F-ratio of 6.21 among all the factors, ranging around ($p < 0.05$) and found statistically significant. The model results specifically F ratio values around 5.65, while the low probability value ($p < 0.05$) and lack of fits showed its high relevance and indicated that it was appropriate for the current investigation related to synthesis and characterization of studied nanoparticles, as also reported earlier[41–43]. Overall, the ANOVA validated the model, with significant F-values indicating that the model parameters were statistically significant. The low *p* values ($p < 0.05$) suggested that the effects of the variables were not due to random chance. Additionally, the lack of fit tests showed non-significant values, indicating that the model fit the data well without significant deviation.

Table 1
Effects and Significance of the experimental variables on response variable (absorbance).

Source	Term df	Error df	F-value	*p* value
Whole plot	2	15.00	3.77	0.0472
Subplot	7	15.00	13.72	< 0.0001
A-Salt	1	15.00	6.21	0.0249
B-Temperature	1	15.00	12.19	0.0033
C-pH	1	15.00	46.38	< 0.0001

The fitness of the experimental model was observed from the normally distributed residuals plot (Fig. 3A and B). The plot indicates that the residuals lie close to the diagonal reference line, and the model proposed that ZnO-NPs are suitable for experimental data application and process interpretation in this study. The threshold value is between − 4.146 to 4.146. (Fig. 3C, D, and E), where the standard residuals should fall. The presence of any error in the model usually indicates the occurrence of the residuals outside the threshold value [41–43]. The current model consistency with their experimental data was

shown as many residuals lie within the threshold value. The residuals and predicted values (Fig. 3C) lie very close to the straight zero error line and show a strong connection to the model parameters. The perturbation plot as shown in (Fig. 3F) presents that three significant independent variables, pH affect the value of R1 more than temperature and salt concentration. Hence, pH produced the maximum effect on the absorbance, followed by temperature and salt concentration on the studied nanoparticles, as reported earlier [41–43].

A circular contour plot indicates no substantial mutual interaction between the associated variables [41–43]. On the contrary, in the present study, the presence of elliptical or distorted plots in Fig. 4 (A, C, and E) serves as empirical support for substantial interactions. The significant interactions for each response variable are exclusively presented and individually described. The contour and 3D plots showed that absorbance increased at higher temperatures and salt concentrations (Fig. 4A). The maximum absorbance (0.9 a.u.) was observed at a salt concentration of 2 g and 37°C. Additionally, increased pH and salt concentration led to a rise in absorbance. As shown in Fig. 4C, the highest absorbance, measured at 1 a.u., was noted at a salt concentration of 2 g and a pH of 7. In contrast, the lowest absorbance, 0.4 a.u., occurred at a salt concentration of 1.5 g and a pH of 5.5.

The findings indicate a positive correlation between pH and temperature and the observed increase in absorbance in the optimization of the studied nanoparticles. According to the data presented in Fig. 4E, the absorbance reached its highest value (1.22 a.u.) at a pH of 7 and a temperature of 37℃. Conversely, the lowest absorbance (0.4 a.u) was seen at a pH of 5.5 and a temperature of 25℃. The pH affects the value of R1 (absorbance) more than temperature and salt concentration. The pH affects the physicochemical properties of ZnO-NPs, including size, shape, and surface charge, which in turn influence their reactivity and molecular interactions [49, 57]. A controlled pH environment ensures the stability and uniformity of nanoparticles, thereby enhancing their efficacy, especially in applications like antibacterial treatments where the interaction with bacterial membranes and genetic material is crucial [58, 59].

The synthesis of ZnO-NPs was influenced by several variables, including the concentration of zinc acetate, the pH of the solution, and the reaction time. The CCD allowed for the systematic evaluation of variables, such as the concentration of zinc acetate, the pH of the solution, and the reaction time, and their interactions. The fit statistics, including standard deviation, mean, coefficient of variance, and regression coefficient, indicated a good fit between the experimental data and the predicted values (Std. Dev. 0.1686, R^2 0.8722, Mean 0.6628, Adjusted R^2 0.7640, C.V. % 0.0631). The high R^2 value (0.8722) suggests that the model can explain a significant proportion of the variability in the response variable (absorbance). Our analysis aligned with (74–76, who highlighted the importance of optimizing key variables such as concentration, pH, and reaction time in the synthesis and application of ZnO nanoparticles. For example, Mahamuni et al. (2019) [76] employed CCD to systematically evaluate the interaction between the variables affecting ZnO nanoparticles and found a good fit between the experimental data and the predicted values, suggesting a strong predictive capability of their mode. Similarly, Asghari et al. (2014) [74] used CCD to optimize the synthesis and adsorption of ZnO

nanoparticles. They reported the critical influence of variables such as concentration, pH, and reaction time on the synthesis of ZnO nanoparticles. Our results agree with these studies in the importance of using CCD to effectively optimize key variables, resulting in high predictive accuracy and robust models for ZnO nanoparticle synthesis and their applications.

3.2. Characterization of nanoparticles

3.2.1. UV-Visible analysis of zinc oxide nanoparticles before and after encapsulation

UV-Vis spectra for ZnO-NPs of pine needles extract were determined using a (U-2800) spectrophotometer within the range from 200–800 nm by using UV-Vis spectra scan with a speed of medium 2400 scan. The maximum absorbance of Zinc oxide nanoparticles (2.7 a.u.) peak value was absorbed at 369 nm and the UV-Vis spectra for ZnO-NPs chitosan encapsulation showed excitation at 355 nm (Fig. 5A and 5B), aligned with the previous study that showed that the absorption peak for ZnO-NPs should be in the region of 300–400 nm [53, 60, 61].

3.2.2. Fourier transforms infrared spectroscopy of *P. roxburghii* ZnO-NPs extracts before and after encapsulation

The FTIR measurements showed the presence of the broad peak at different wavenumbers correspond to their corresponding functional group in phenolic, alcoholic, and other compounds. FTIR spectra were obtained using the KBr pellet method, with dried nanoparticle powder mixed with spectroscopic-grade KBr at a 1:100 ratio and pressed into discs for analysis. The band assignments were cross-verified with published spectral data on ZnO and chitosan-based nanocomposites. The strong alkyl C-H stretching vibrations of methyl groups are present at 2900 cm^{-1}. The occurrence of distinct peaks *P. roxburghii* ZnO-NPs extracts at various wavenumbers can be ascribed to the elongation of carbon-hydrogen (C-H) bonds within the alkane functional group. The existence of absorption peaks at 489 cm^{-1} of *P. roxburghii* extracts (Fig. 5C) indicates the presence of ZnO-NPs. *Pinus. roxburghii* extracts showed absorption peaks at 800 cm^{-1} that suggest the presence of aromatic compounds, as reported previously [54]. FTIR spectra for ZnO-NPs encapsulated chitosan powder by microwave-assisted encapsulation showed the interaction between chitosan and the guest molecule (ZnO-NPs) by modifying the spectral properties after encapsulation. The peak at 3200–3400 cm^{-1} (Fig. 5C) could be attributed to stretching the O-H groups in alcohols and O-H groups in the aromatic ring of phenols [27, 53, 54, 61, 62]. The firm peaks (Fig. 5D) for chitosan at 1580–1650 cm^{-1}, which is used for encapsulation, are due to the medium stretching of the N-H bonds. N-H bending was observed in the ZnO-NPs encapsulated in chitosan powder at 1580–1650 cm^{-1}. The sharp peaks (Fig. 5C) at 1400 – 1300 cm^{-1} are assigned to the C-H stretching of the alkanes group. The absorbance of IR peak (Fig. 6A) at 1020 and 1062 to 1150 cm^{-1} could be assigned to the C-O stretching of the COOH groups and C-O-C bending, respectively. The broad peak (Fig. 5C) was observed centered at 450–500 cm^{-1} in the fingerprint region for the ZnO-NPs, which is the fingerprint region according to previous research [53, 60, 61].

Besides nanoparticles, carbon nanomaterials, such as carbon nanotubes (CNTs) and fullerene, are considered potential antibacterial agents, in addition to their other uses. Carbon nanotubes discharge carbon into the environment and might exert higher toxicity effects compared to nanomaterials, and their negative environmental effect is scarce [80. Therefore, in this study, we synthesized ZnO nanoparticles to observe the antimicrobial activity of *P. roxburghii* dry needles extract against citrus canker-causing bacteria. ZnO nanoparticles have gained considerable attention due to their non-hazardous, eco-friendly nature [81], biocompatibility [82], and lower-cost synthesis procedures. They exhibit selective toxicity against targeted bacteria [83] and serve as an essential trace element of the human body [84].

Various conventional methods are used to control plant microbial diseases, but these techniques often have adverse environmental effects on plants and other organisms. In this research, the use of nanoparticles has emerged as a safer, eco-friendly method for plant microbial disease management and acquiring disease resistance in crops compared to conventional methods. Research has recently found that ZnO-NPs synthesized from *P. roxburghii* extracts have antimicrobial potential for acquiring resistance against gram-positive bacteria using[63–65]. Our study corroborated these findings, showing promising results in resistance against both gram-positive and gram-negative plant pathogens, such as *X. citri*[66].

By modifying the synthesis conditions of ZnO-NPs, such as pH, these nanoparticles can be engineered to interact specifically with and neutralize targeted pathogens. Bai et al. [89] highlighted the antimicrobial efficacy of ZnO nanoparticles synthesized under different conditions, including pH, demonstrating their ability to interact with and neutralize pathogens. The use of ZnO-NPs minimizes environmental impact and reduces the risk of affecting non-target species. Such a targeted approach enhances disease control and aligns with sustainable agricultural practices by reducing the overall use of harmful chemicals [90]. The successful application of ZnO-NPs against gram-negative bacteria underscores the broad-spectrum potential of nanomaterials in plant disease management, opening new avenues for developing tailored nanoparticle-based solutions for various types of pathogens [91].

3.2.3. X-ray diffraction (XRD) analysis of pine needles powder, chitosan and zinc oxide nanoparticles before and after encapsulation

The XRD pattern data indicated that nanoparticles synthesized in this study exhibited peaks that signify the presence of precursor material. The ZnO-NPs showed distinct peaks located at 2θ = 22.24, 24.64, and 50.38. The XRD pattern of ZnO-NPs, manufactured by using a green technique, was obtained within the range of 20–80 degrees (20° <2Θ >80°) and the patterns when we generated a ZnO-NP were matched and compared the XRD pattern with the reference PDF card (01-070-2551) of zinc oxide[10]. Prominent diffraction peaks identified in the XRD spectra of ZnO-NPs confirmed a hexagonal Wurtzite crystal structure. The narrow and sharp peaks (Fig. 5G and H) in the XRD spectrum indicated the high purity and crystalline nature of the synthesized nanoparticles, both with and without encapsulation. The crystallinity index is about 54%.

The XRD analysis also noted the amorphous nature of the pine needles' powder and chitosan powder (Fig. 5E and 5F). The degree of crystalline of the nanoparticles, both with and without encapsulation, was higher than that of the pure chitosan powder and pine needle extract powder. The XRD results are consistent with those reported by Govindan et al.[67] in their work on chitosan–silver nanocomposite materials. The peaks for encapsulated ZnO-NPs were observed at 2θ = 20.08, 22.75, 26.38, 30.36, 33.23, 35.28, 38.48, 39.39, 41.37 and 48.63, confirming that encapsulated ZnO-NPs were produced in Wurtzite structure, in alignment with the literature [53, 60, 61].

3.2.4. Dynamic light scattering (DLS)

Dynamic light scattering illustrates the size distribution graph of ZnO-NPs, indicating 68 nm particle size (Fig. 6A). The zeta potential, representing the surface charge of the particles, was measured (e -25.66 Mv) as shown in Fig. 6B. Dynamic light scattering stands out as one of the extensively utilized methods for determining the size of nanoparticles in a colloidal form. Technically, the size of nanoparticles measured using DLS is calculated from the metal core of the nanoparticles to the layer surrounding them, leading to a larger size estimation for the nanoparticles[68].

3.2.5. Morphological features of ZnO-NPs using SEM analysis

The SEM images of the synthesized nanoparticle showed that the particles were granular in nature and spherical in shape (Fig. 7A, B, C). The particles' agglomeration was observed from the SEM image (Fig. 7C) at a higher resolution, possibly due to aging [54]. The structural morphology was also obtained for the microwave-assisted encapsulation nanoparticles through SEM. The SEM micrographs of ZnO-NPs, encapsulated in chitosan powder, at 9.99 X, 5 KX, 1.92 KX and 129 KX (Fig. 8A-D) magnification clearly exhibited that the size of the encapsulated powder by performed by the microwave-assisted method, was larger than nanoparticles without encapsulation. The encapsulated nanoparticles' surface morphology was clearly seen with magnification at 129 X. The surface of the encapsulated ones obtained by the microwave-assisted encapsulation method was smooth[53, 60]. Although SEM provided valuable morphological insights, limitations in magnification restrict visualization of particles < 100 nm. Future studies should incorporate TEM for precise nanoscale imaging.

3.3. Antimicrobial activity determination

The oil obtained through the traditional heat distillation method from *P. roxburghii* wood was tested against *X. citri* by using the disc diffusion method. The anti-bacterial wood oil activity was checked after 24 and 48 hours. A clear zone of inhibition of 16 ± 8.5 mm was observed after 24 hours of incubation, while a 27 ± 4.42 mm zone was recorded after 48 hours (Fig. 9A). The size of the inhibition zone exhibited a positive correlation with the oil concentration, indicating that the oil content increased with the increase of the inhibition zone (Fig. 10A-C). The average inhibition zone of pine needle extracts was 3.30 ± 1.00 mm, as shown in Fig. 9 (B and C). However, the use of ZnO-NPs resulted in a higher zone of inhibition of 7.30 ± 1.60 mm and suggested that the synthesized nanoparticles enhanced the antimicrobial activity and could be useful in protecting plant citrus canker disease ((Fig. 9B). Table 2).

The measurement of inhibitory zones was conducted on the first and second emulsions of encapsulated nanoparticles, which were prepared using the lyophilization technique. Following the initial emulsion, the nanoparticles exhibited a zone of inhibition measuring 7 ± 1.40 mm (as shown in Fig. 9C). After the second emulsion, the zone of inhibition decreased to 4 ± 2.80 mm (Fig. 9C). In contrast, the zone of inhibition resulting from the simple needle extracts against the bacteria was 2 ± 0.00 mm. This observation suggested the antibacterial activity was enhanced after encapsulation through lyophilization, leading to increased stability of the nanoparticles, as reported earlier [53, 60, 61] (Table 2).

The results obtained from the experiment demonstrate that the microwave-assisted encapsulated nanoparticles exhibited larger zones of inhibition, measuring 18.50 ± 3.40 mm (Fig. 9D and E). In comparison, the nanoparticles without encapsulation displayed zones of inhibition measuring 15 ± 4.20 mm (Fig. 9D and E). Additionally, the encapsulated simple pine needle extract exhibited zones of inhibition measured 1 ± 0.50 mm (Fig. 9D and E). These findings suggest that the encapsulation of nanoparticles using the microwave-assisted method significantly increased in the inhibition zones (Fig. 9F, Table 3).

Table 2
Antimicrobial potential of *P. roxburghii* wood oil, both encapsulated and unencapsulated needles extract, and ZnO-NPs against *X. citri* (n = 6).

Encapsulation method	Sample	Inhibition zone (mm)
Without encapsulation	Pinewood oil	12.70 ± 5.50
	Pine needle extract	3.30 ± 1.00
	ZnO-NPs	7.30 ± 1.60
Encapsulated via lyophilization	Needles extract	2.00 ± 0.00
	NPs after the first emulsion	7.00 ± 1.40
	NPs after the second emulsion	4.00 ± 2.80
Encapsulated through Microwave-assisted method	Needles extract	1.00 ± 0.50
	NPs without encapsulation	15.00 ± 4.20
	Encapsulated NPs	18.50 ± 3.40

Two different concentrations of nanoparticles have been tested to examine the variation in their antibacterial efficacy. The pine needle extracts containing 2 mg/ml nanoparticles exhibited a smaller zone of inhibition (2 ± 0.00 mm), than 4 g/ml nanoparticles (4 ± 0.00 mm). The technique was executed thrice, yielding consistent outcomes in each instance. The results demonstrate that augmenting the concentration of nanoparticles leads to a corresponding enhancement in their antibacterial efficacy (Fig. 10 BC and D) (Table 3).

Table 3
Comparison of antibacterial activities (means ± SD) of pine wood oil and ZnO-NPs (n = 3) at different concentrations against *X. citri*.

Inhibition zone of *X. citri* (mm)					
Pinewood oil		2 mg/ml Nanoparticles		4 mg/ml Nanoparticles	
24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
12.00 ± 2.80	24.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
Each constituent's value consisted of ± SD (n = 3)					

3.2. Broth dilution

Based on Table 4, the MIC of ZnO-NPs for *X. citri* was estimated to be 0.5 mg mL^{-1}, as lower doses were not tested. Since no live cells were identified after 48 hours, the Minimum Bactericidal Concentration (MBC) for *X. citri* was determined to be 1.0 mg mL^{-1}. For the investigated ZnO-NPs concentrations, the bacterial concentration increased comparably to the negative control. Concentrations of more than 2 mg mL^{-1} were not investigated due to considerable ZnO precipitation, which impacted the reproducibility of the results.

3.3. Minimum Bactericidal Concentration/Minimum Inhibitory Concentration

Microbial growth curve

After 24 hours, no viable cells of *X. citri* were detected when the MBC was applied in this work. However, the negative control reached the maximum cell count of approximately 10^9 CFU mL-1 (Fig. 2B). After 6 hours of incubation, ZnO-NPs at MBC reduced bacterial count by 40% and 60% compared to the negative control for the Gram-negative phytopathogens *X. citri*. It indicates that ZnO-NPs can be more effective against *X. citri*. The growth curves at MIC presented a reduction in the cell count in the first 24 hours, followed by an increase, reaching counts close to the initial cell count of microorganisms. At 48 hours, ZnO-NPs had a mild influence; the bacterial cell counts were reduced compared to the negative control at all periods. Interestingly, the antibacterial effect of ZnO-NPs was more effective against *X. citri* when bacterial growth was monitored for 80 hours under the impact of MIC of ZnO-NPs applied to *X. citri*(Fig. 2B).

The initial decline resulted from the microorganism's vulnerability to ZnO-NPs, which decreased total concentration. As a result, some microorganisms likely grew over a long period due to variation in phenotype within the microbial community, leading to the formation of separate groups of organisms. Microbial populations utilize the formation of variant subpopulations to better withstand stressful situations, such as the presence of ZnO [69]. This bacterial behaviour towards studied nanoparticles highlights the time-dependent aspect of the MIC method, suggesting that experiments with prolonged incubation durations are likely to be the cause of varied MIC values.

Table 4
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of zinc oxide nanoparticles (ZnO-NPs) against *X. citri*.

ZnO Nanoparticles (mg ml^{-1})				MIC/MBC		
0.50	1.00	1.50	2.00	Negative control	MIC	MBC
0.05	0.91	0.98	1.20	1.76	0.50	1.00

The nanoparticle encapsulated using either lyophilization or microwave-assisted methods in the present research showed antibacterial properties. However, it was observed that the nanoparticles encapsulated through the microwave-assisted method exhibited higher antibacterial activity than those encapsulated through the lyophilization or freeze-drying method. In a previous study conducted by [70]. It was found that nanoparticles encapsulated through a microwave-assisted method demonstrated better antibacterial activity against both Gram-positive and Gram-negative bacteria than those encapsulated through lyophilization or freeze-drying. This finding is consistent with the results of the current study. This enhanced antimicrobial performance highlights the effectiveness of microwave-assisted encapsulation in bolstering the bioactive properties of ZnO-NPs.

The results of the current study indicated that antibacterial agents can be synthesized more efficiently through microwave-assisted encapsulation of nanoparticles due to their technical advancement and environmentally friendly nature, making it a step forward in sustainable agricultural practices. Higher antibacterial efficacy against pathogen *X. citri*. showed that the encapsulation process has increased the stability and delivery of active compounds, resulting in superior performance compared to non-encapsulated extracts, and seems effective and sustainable disease management strategy for sustainable agriculture [71, 72]. In addition, the encapsulation of nanoparticles in chitosan has further enhanced their antibacterial properties and provides a potent solution for combating plant diseases, such as *X. citri*, supporting healthier crop growth, and enhancing the profitability of farming ventures.

Microwave-assisted encapsulation techniques, as reported earlier, have been found to use nanoparticles with exceptional antibacterial activity against *X. citri*, as opposed to conventional lyophilization methods, offering a promising innovation for plant disease management [70]. This approach not only boasts higher efficiency and yield but also aligns with the growing call for sustainable agricultural solutions. Additionally, the study's use of non-wood materials such as dried pine needles for nanoparticle synthesis offers a solution for waste management while reducing wildfire risks and curbing deforestation, all of which contribute to environmental conservation [44]. The characterization techniques have validated the structural integrity and functional efficacy of the synthesized ZnO-NPs. The successful use of the nanoparticles in the present work, particularly in combating gram-negative pathogens, highlights the potential of nanotechnology to develop eco-friendly and effective solutions for plant disease management, while, according to previous studies, nanoparticles have high antibacterial properties against gram-positive than gram-negative [17]. This approach can potentially overcome the limitations of conventional methods that pose environmental hazards, as noted by [66].

The present study synthesizes the ZnO-NPs from the needles of *P. roxburghii*, which is the largest non-wood source of pine plant. This approach avoids using the plant's stem and root oil, which is highly labor-intensive and contributes to deforestation [13]. The green technology applied in this research is safer than other chemical-based methods (such as eradication, elimination, and using copper spray) for the synthesis of antimicrobial products [73–76]. These chemical methods are costly, labor-intensive, toxic to the environment, and challenging for non-commercial growers to reduce microbial pathogens [77].

Future Applications and Research Limitations

The results of this study have important implications for the sustainable management of plant diseases, especially in the agricultural sector. The use of *P. roxburghii* needle extracts to synthesize zinc oxide nanoparticles (ZnO-NPs) and encapsulate them in chitosan shows promise for future applications. This method could be used to create a variety of broad-spectrum antimicrobial agents effective against different plant pathogens, potentially transforming plant disease management by reducing reliance on chemical pesticides and minimizing environmental impacts. These nanoparticles could help control diseases like citrus canker, leading to increased crop yields and better fruit quality, which is critical for global food security. The environmentally friendly approach of green synthesis is in line with sustainable agricultural practices, promoting eco-friendly solutions for crop protection. Moreover, the biocompatibility and antimicrobial properties of chitosan-encapsulated ZnO-NPs offer potential applications in pharmaceuticals and biomedicine as new therapeutic agents. Further research into their use as nano-fertilizers or soil amendments could improve soil health and plant growth, supporting sustainable farming practices.

The study has some limitations that need to be acknowledged for a balanced understanding. First, there is a lack of comprehensive toxicity studies for chitosan and ZnO-NPs. Conducting extensive toxicity assessments on various plant and animal systems is crucial to ensure their safety for widespread agricultural use. Additionally, the synthesis and characterization of nanoparticles require specific equipment and resources that may not be readily available in all research settings, potentially limiting the study's replicability. Another concern is the potential environmental impact of large-scale production and application of these nanoparticles, which must be thoroughly evaluated to avoid unforeseen ecological risks. Furthermore, the phytochemical composition of plant extracts can vary based on geographical location, season, and plant age, affecting the consistency and efficacy of nanoparticle synthesis. Lastly, the economic viability of producing and applying these nanoparticles on a large scale needs careful assessment to determine their practical feasibility for farmers and the agricultural industry. Addressing these limitations through future research will be essential for practically implementing these findings.

Conclusions

This research contributes to sustainable agriculture by creating eco-friendly synthesized chitosan ZnO-NPs using extracts from pine needles. Using RSM and CCD, the study identified key factors that affect

nanoparticle synthesis, with pH being the most critical variable affecting nanoparticle synthesis. The use of microwave-assisted encapsulation techniques has enhanced the antibacterial efficacy of nanoparticles against gram-negative pathogens like *X. citri* and represents a step forward from traditional methods such as lyophilization. This progress highlights the potential of ZnO nanoparticles in managing plant diseases, presenting an eco-friendlier alternative to conventional pesticides. Synthesizing nanoparticles from pine needles offers a dual advantage of effective waste management and the creation of an eco-friendly tool for disease management. However, a thorough investigation is required to ensure the safe application of nanoparticles in agriculture due to potential toxicity and long-term environmental impact at high concentrations.

Abbreviations

CCD
Central composite design
DLS
Dynamic Light Scattering
FTIR
Fourier transform infrared spectroscopy
MBC
Minimum Bactericidal Concentration
MIC
Minimum Inhibitory Concentration
NPs
Nanoparticles
P. roxburghii
Pinus roxburghii
RSM
Response Surface Methodology
SD
Standard Deviation
SEM
Scanning Electron Microscope
UV-Vis
Ultraviolet Visible
X. citri
Xanthomonas citri
XRD
X-ray Diffraction

Declarations

Ethics approval: Not applicable

-Consent to Participate We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that all the authors listed in the manuscript have been approved by all of us.

Funding: Not applicable.

-Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

-Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

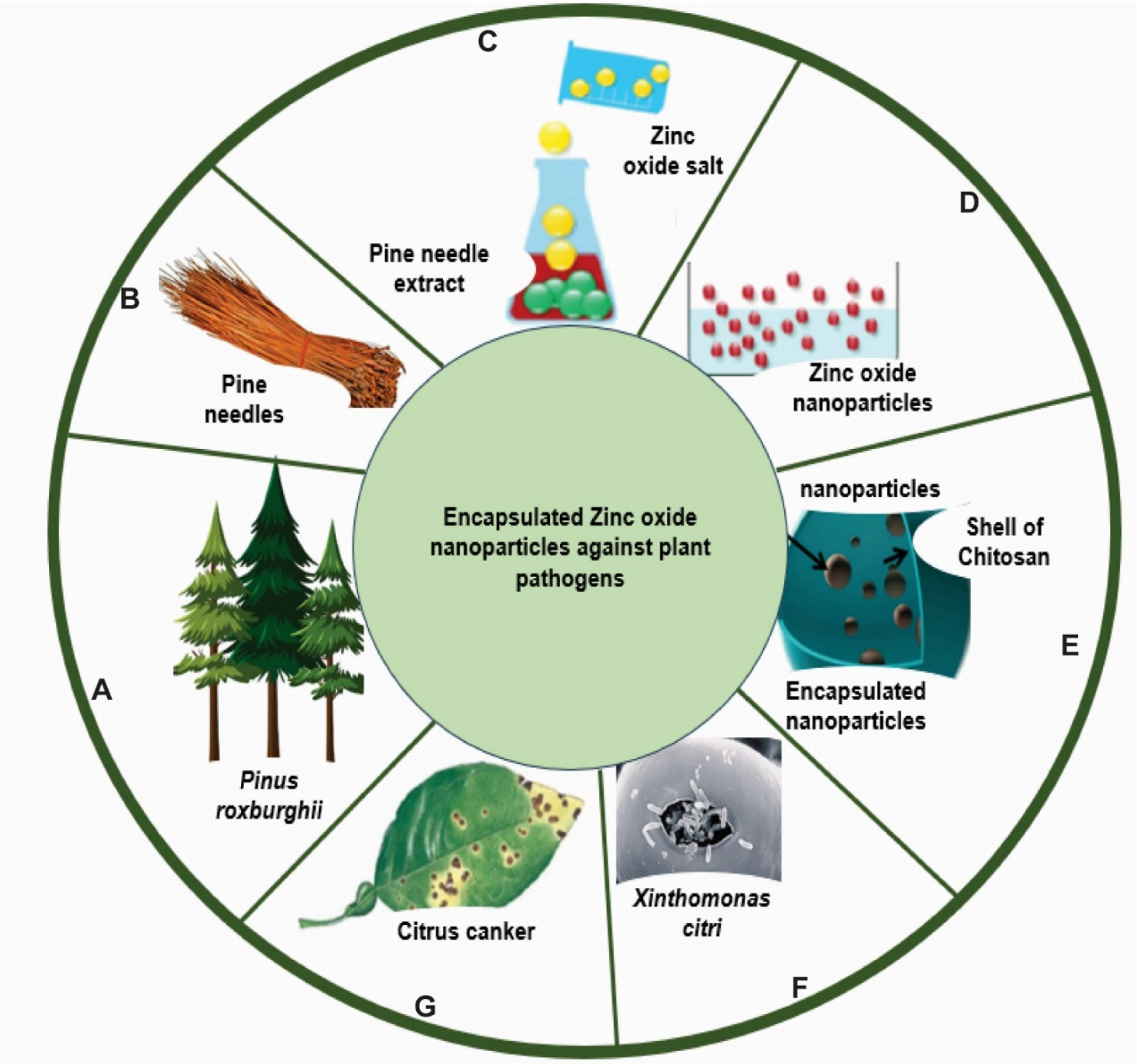

Figure 1

Encapsulated Zinc oxide nanoparticles against plant pathogens (A) *Pinus roxburghii* plant (B) Needles collected from the plant (C) Plant extract and Zinc acetate salt for the nanoparticles preparation (D) Prepared nanoparticles (E) Encapsulate the nanoparticles in the choitsan (F) Check the antimicrobial activity against *X. citri* (G) The citrus cancer causing bacteria collected from the Citrus plant.

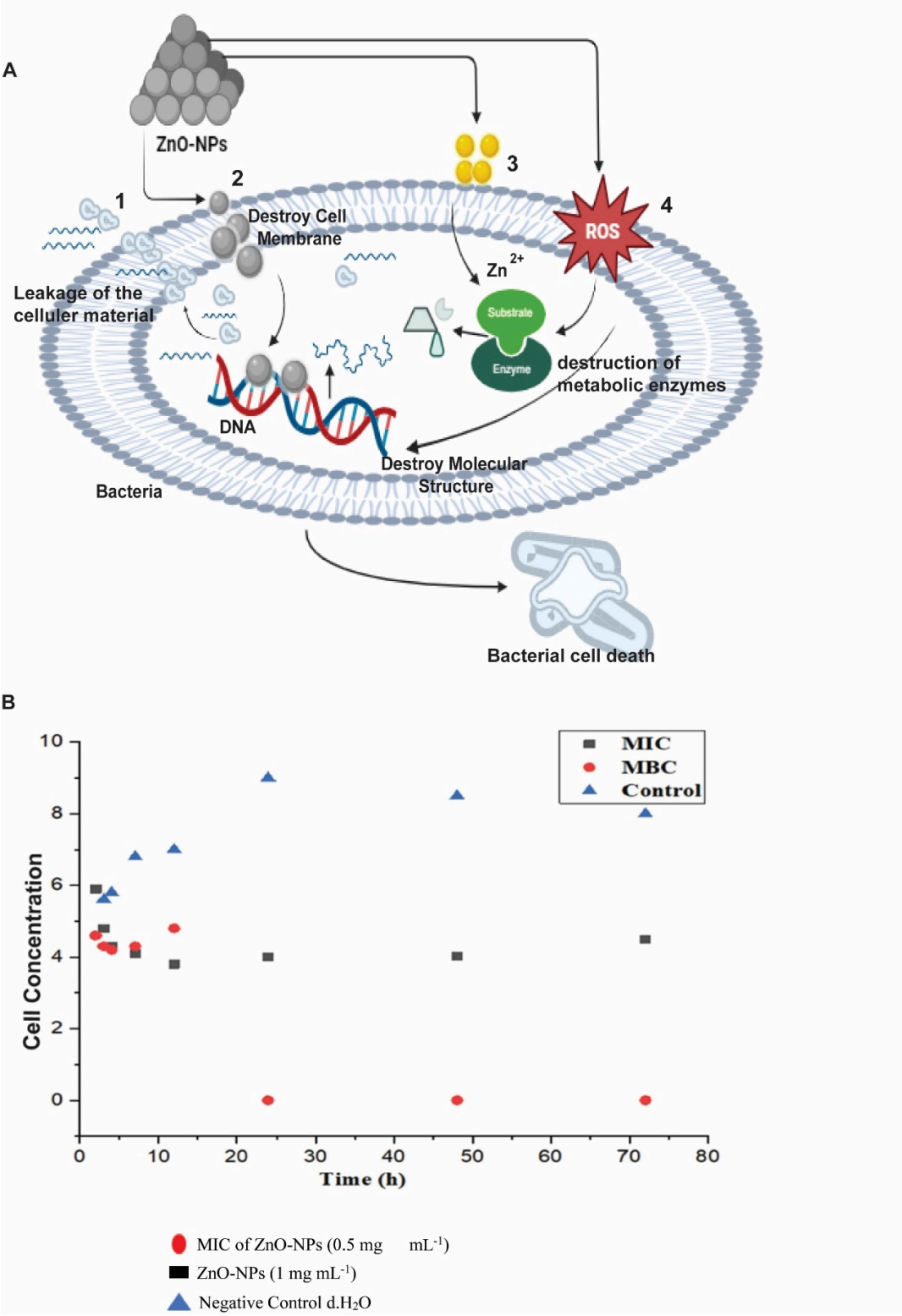

Figure 2

(A) Antimicrobial Mechanisms of Zinc oxide nanoparticles and its interaction with bacteria; 1) Zinc oxide nanoparticles accumolate on the bacterial cell membrane, which lose its integrity; 2) Leakage of the cellular contents due to membrane disruption and lead to bacterial cell death; 3) ZnO-NPs could produced zinc ions, once enter inside the cell it incuporated into the molecular structure (DNA) and inhibit some enzymatic functions which disrupt the bacterial metabolism and cause cell death; 4) ZnO-

NPs generate ROS, with in the bacterial cells ROS produced oxiditive stress, which inhibit some enzymes and destroy molecular structure. **(B)** Cell concentration over time.

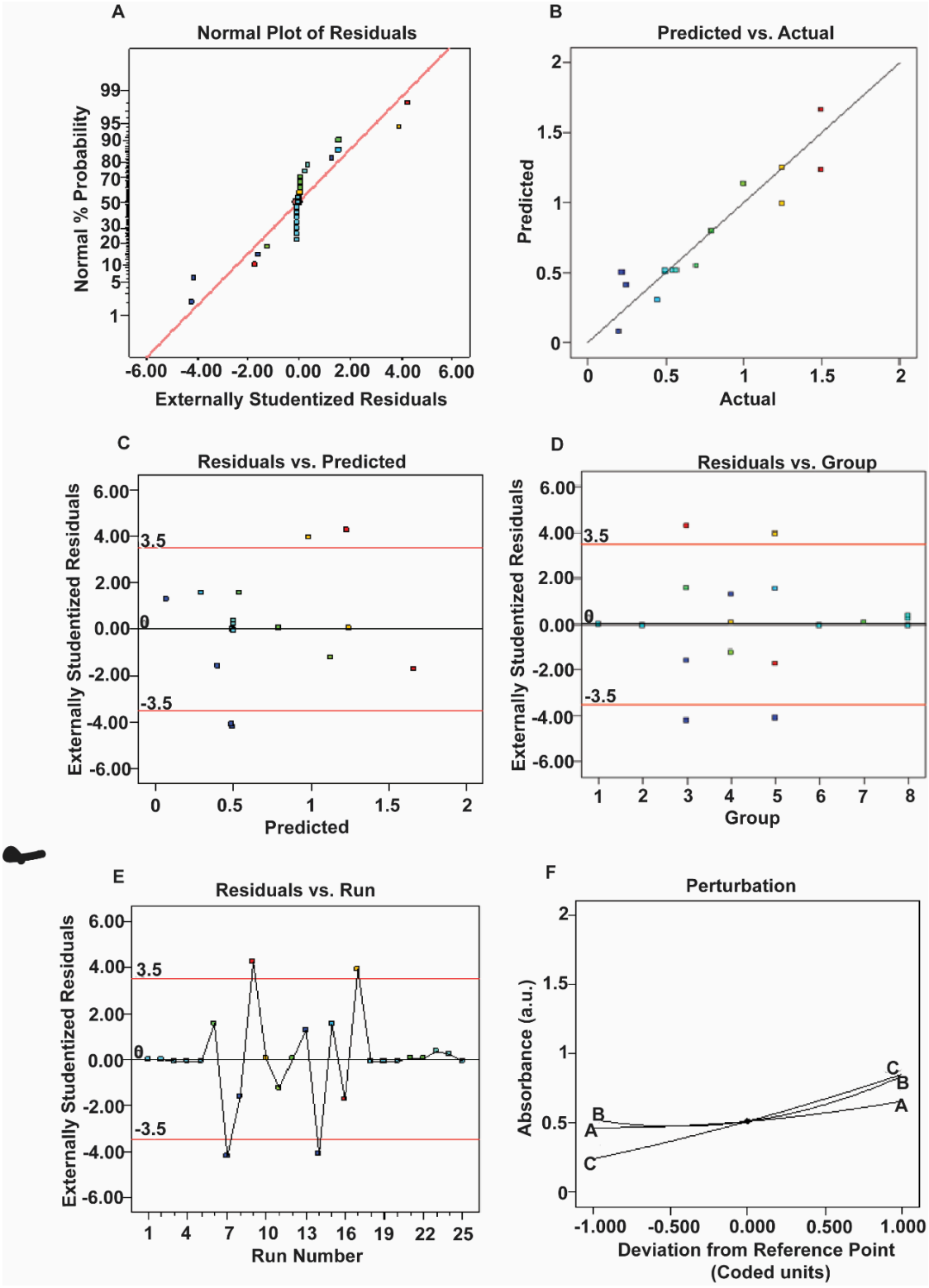

Figure 3

(A) Normal plots of residuals of the absorbance (B) Predicted verse Actual plot (C) Residual verses predicted plot (D) Residual verses Group plot (E) Residual verses Run plot (F) Perturbation plots showing

the effect of all the three factors (A: Salt concentration, B: Temperature, C: pH) on the response variables (R1: absorbance)

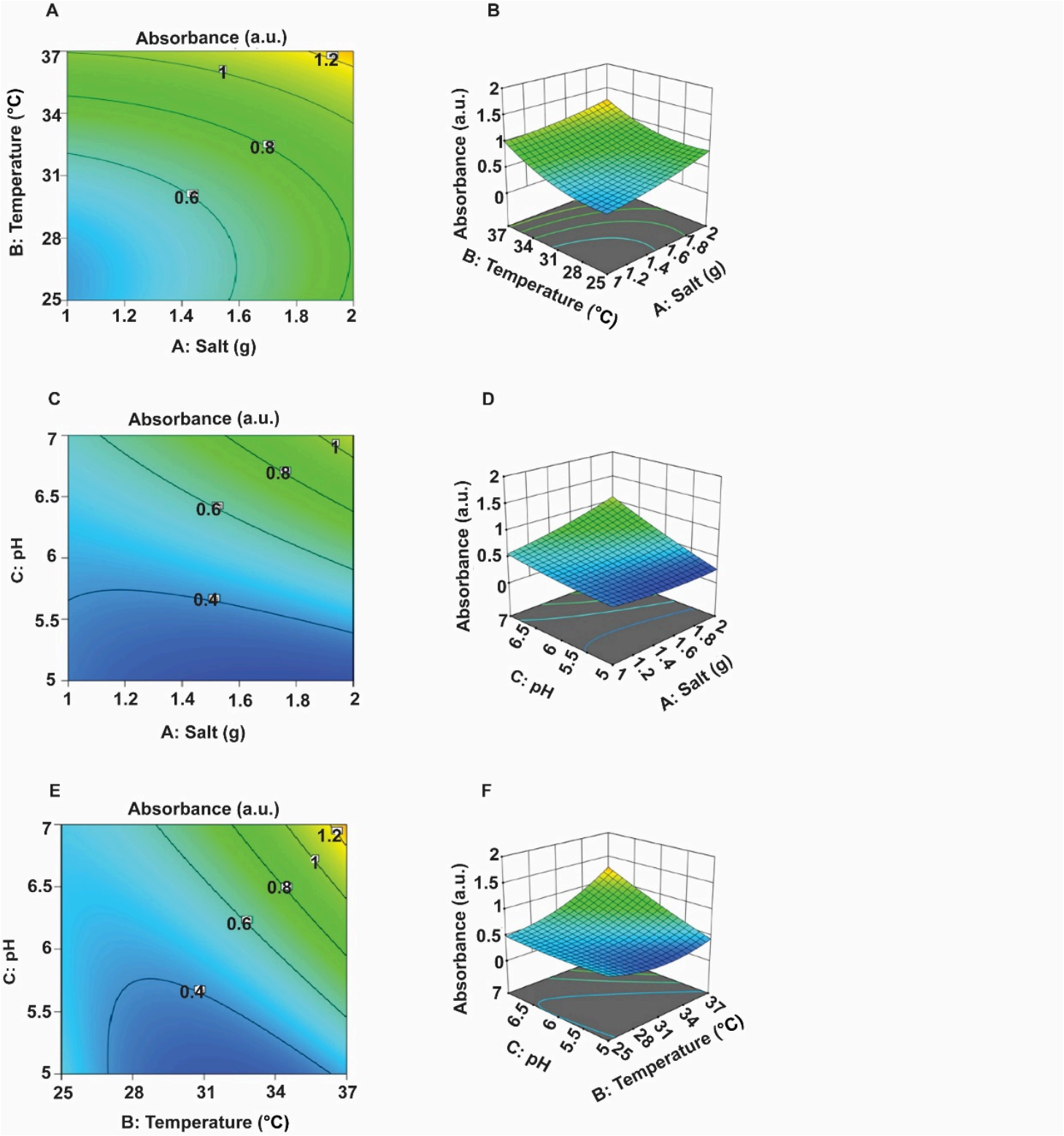

Figure 4

(A) The Contour plot and (B) 3D response surface plots showing the interactive effects of temperature and salt concentration (C) The Contour plot and (D) 3D response surface plot indicates the Interactive

effects of salt concentration with pH (E) contour and (F) 3D response surface plots showing the interactive effects of pH and temperature on absorbance

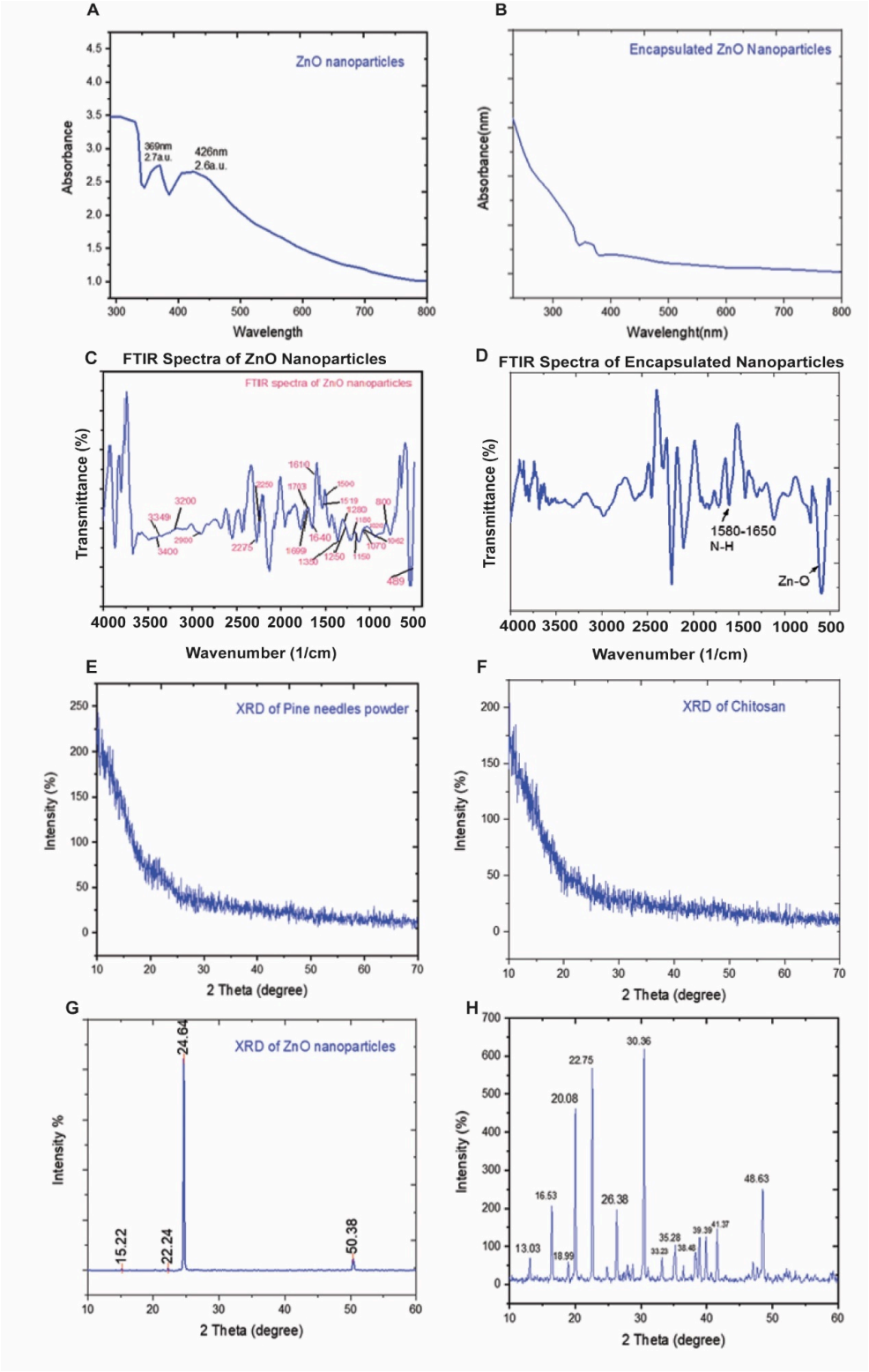

Figure 5

Characterization of ZnO-NPs before and after encapsulation in chitosan (A-B) UV of ZnO-NPs (A) UV of ZnO-NPs before encapsulation (B) UV of ZnO-NPs after encapsulation (C-D) FTIR of ZnO-NPs (C) FTIR of ZnO-NPs before encapsulation (D) FTIR of ZnO-NPs after encapsulation (E-H) XRD of ZnO-NPs (A) XRD

of pine needles powder (B) XRD of Chitosan powder (C) XRD of ZnO-NPs before encapsulation (D) XRD of encapsulated ZnO-NPs.

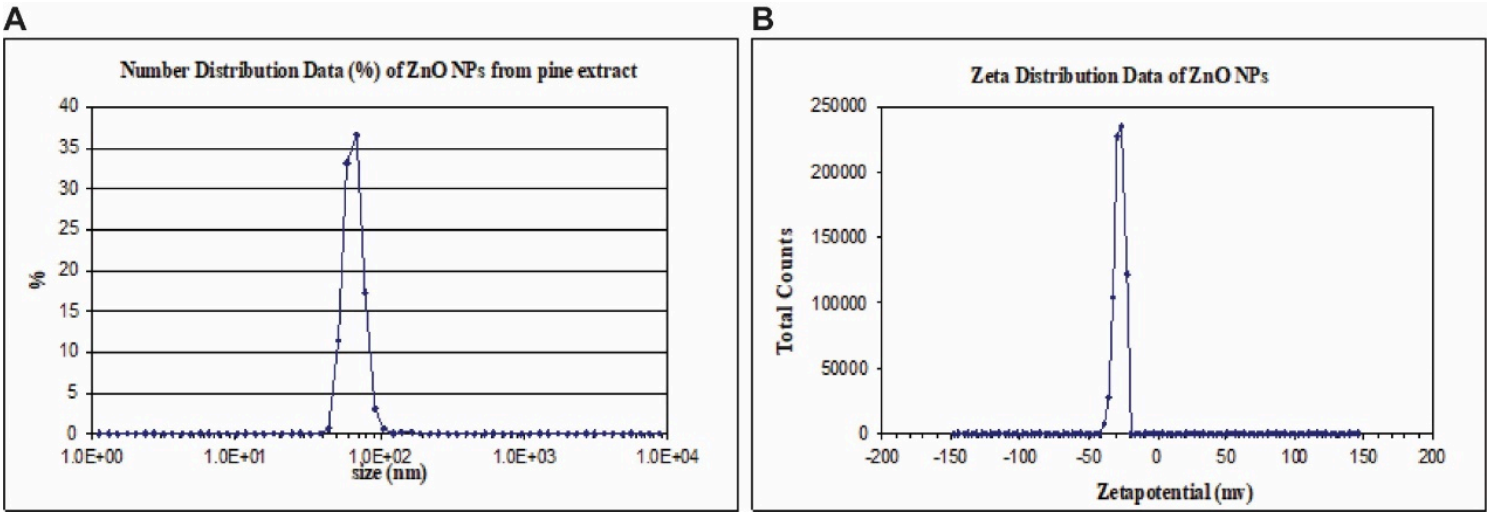

Figure 6

Zeta potentail and Zeta sizer of green synthesized ZnO-NPs (A) size distribution graph of ZnO-NPs for particle size (B) Zeta potential for the surface charge of the particles.

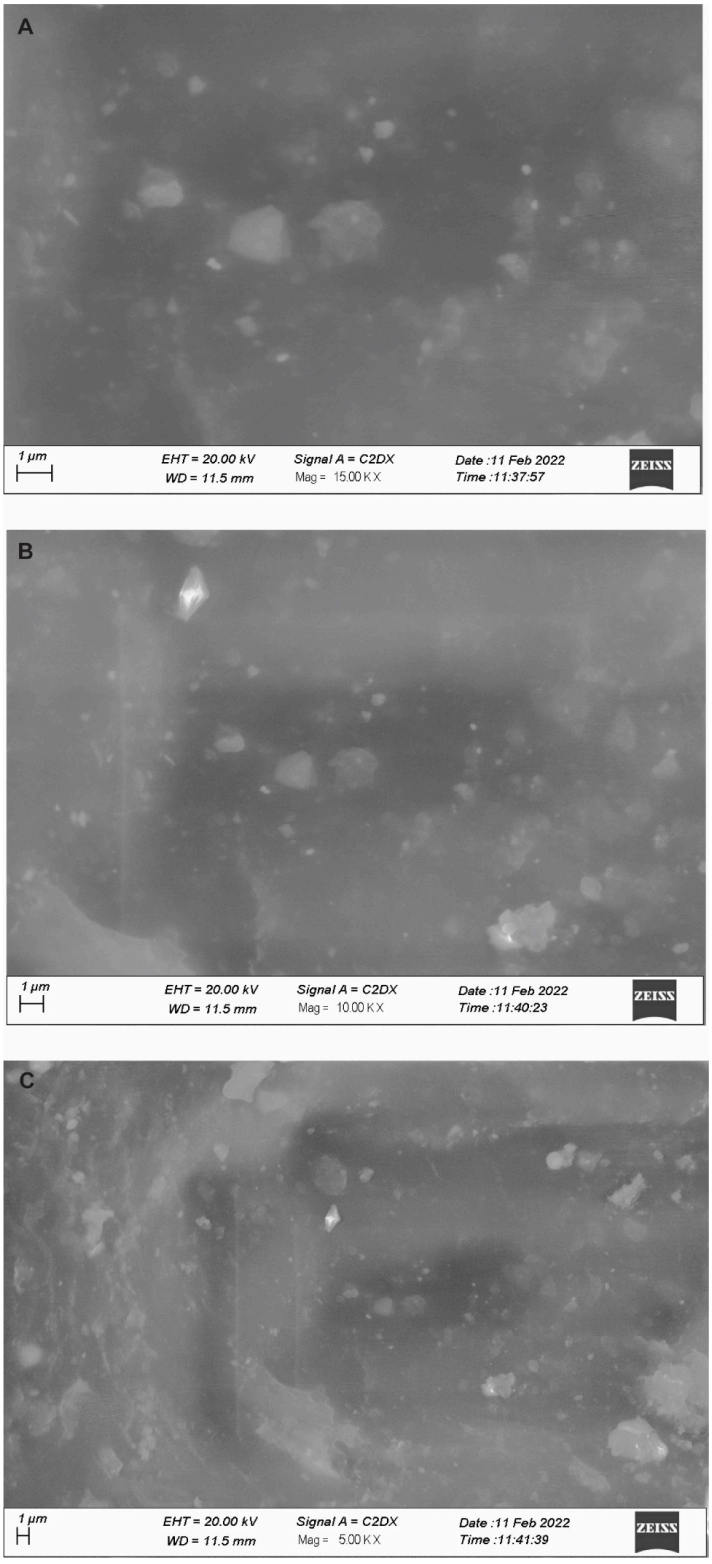

Figure 7

SEM image of Zinc oxide nanoparticles at (A) 15 KX, (B) 10 KX and (C) 5 KX magnification

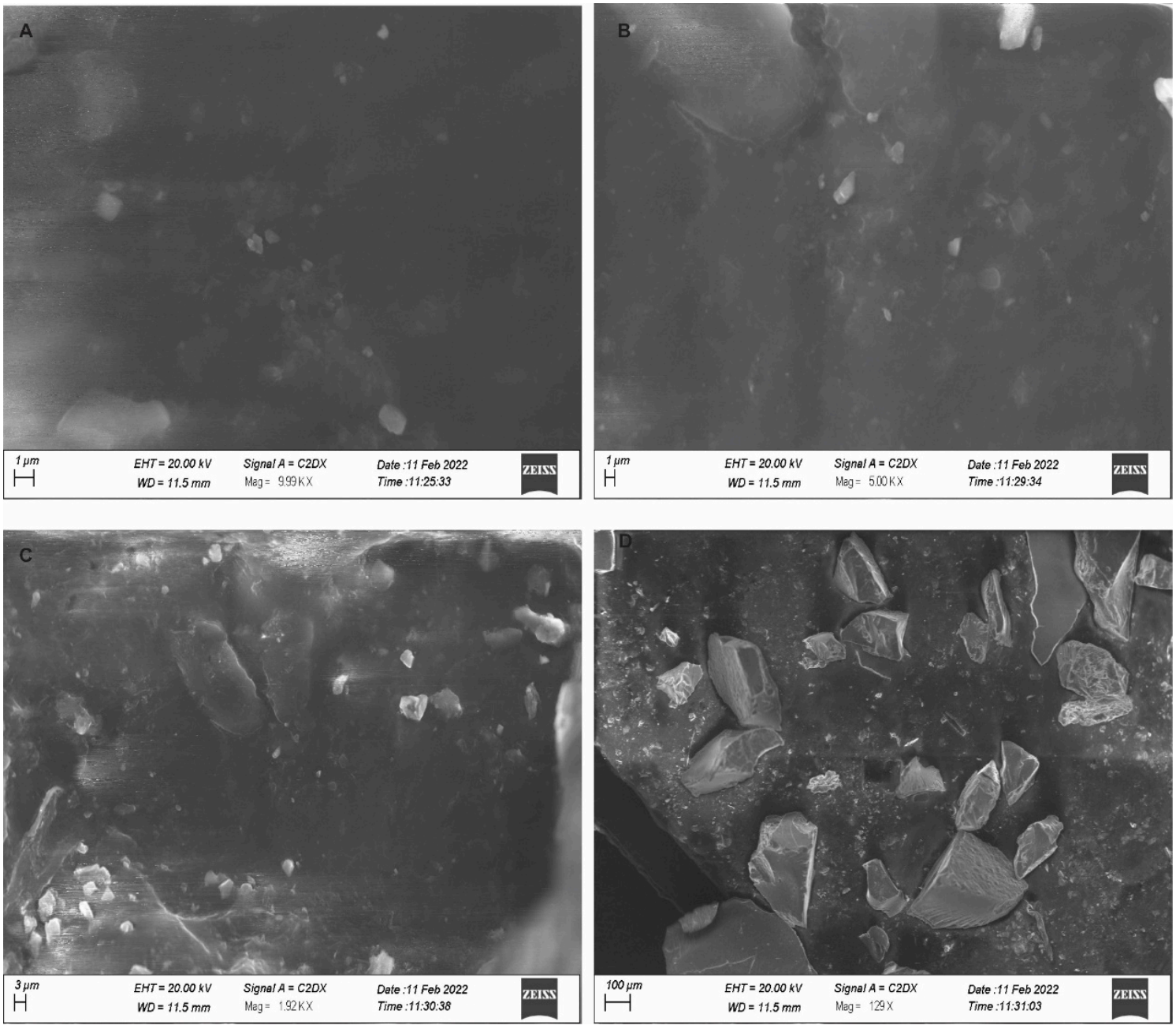

Figure 8

SEM micrograph of ZnO-NP encapsulated in Chitosan at (A) 9.99 KX, (B) 5.00 KX, (C) 1.92 KX and (D) 129 X magnification.

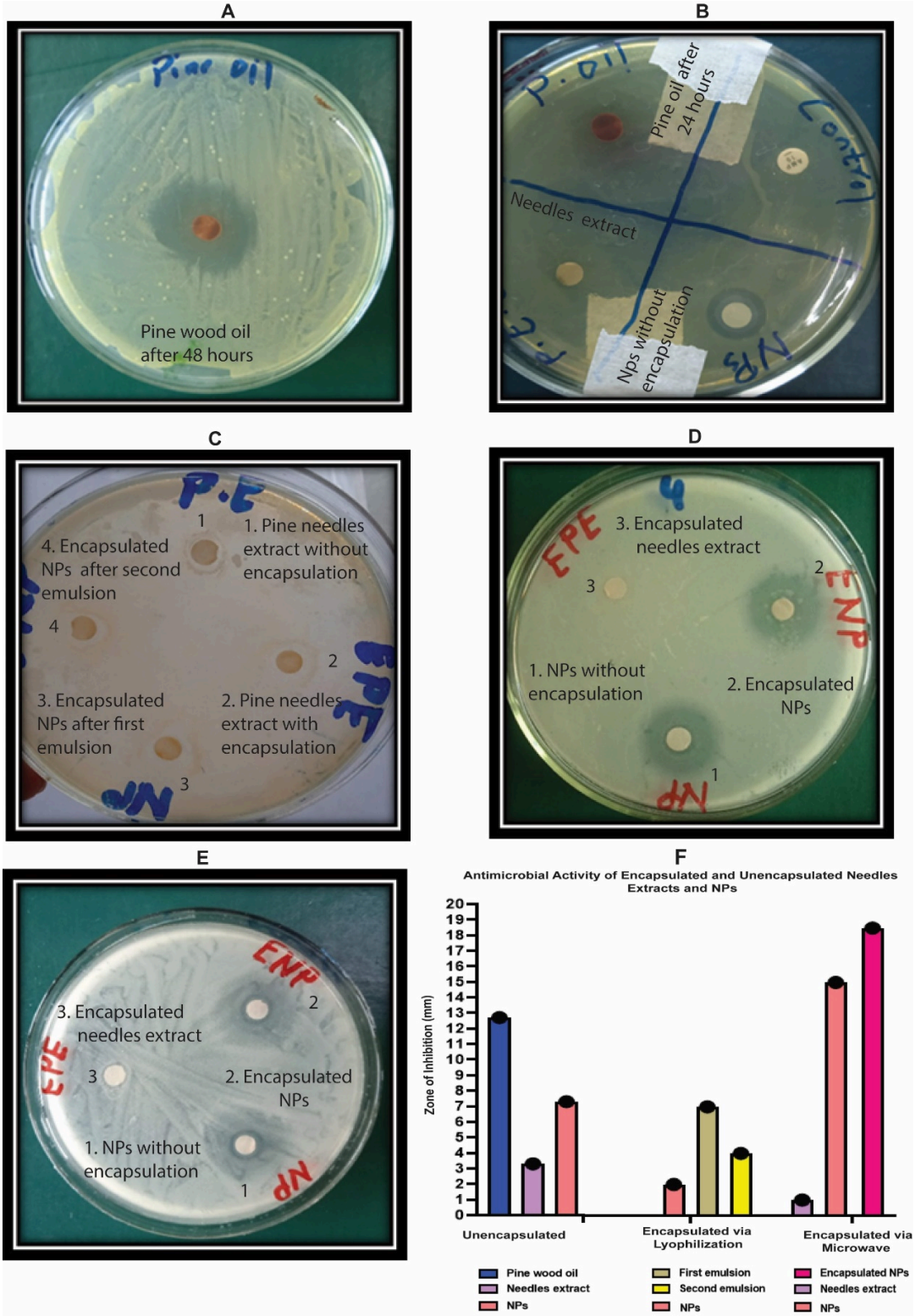

Figure 9

(A) Antibacterial activity of pine wood oil (B) Zinc oxide nanoparticles of pine needles extracts without encapsulation (C) nanoparticles encapsulated through lyophilization method (D and E) nanoparticle encapsulated via microwave assisted method; NPs nanoparticles, EPE encapsulated plant extracts, PE plant extracts and ENP encapsulated nanoparticles (F) Statistical analysis of bacteriostatic zones of inhibition.

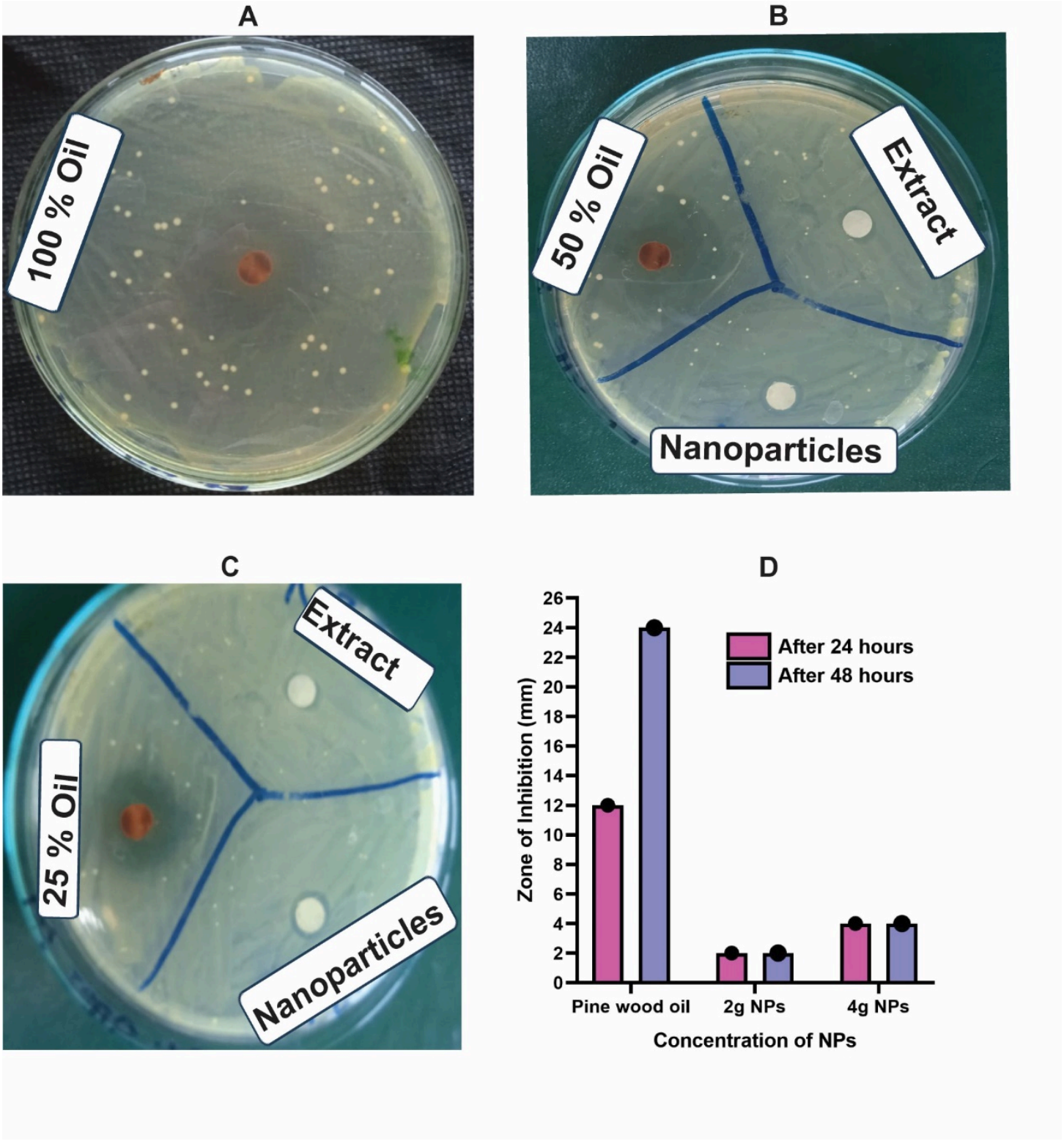

Figure 10

Antimicrobial activity of *Pinus roxburghii* oil and nanoparticles (NPs) at different concentrations (A) 100% oil; 10 µL (B) 50% oil; 5 µL (C) 25% oil; 2.5 µL. The anti-bacterial potential of the oil increase at increasing concentration (D) Statistical analysis of zone of inhibitions at different concentrations of oil and NPs and time interval.

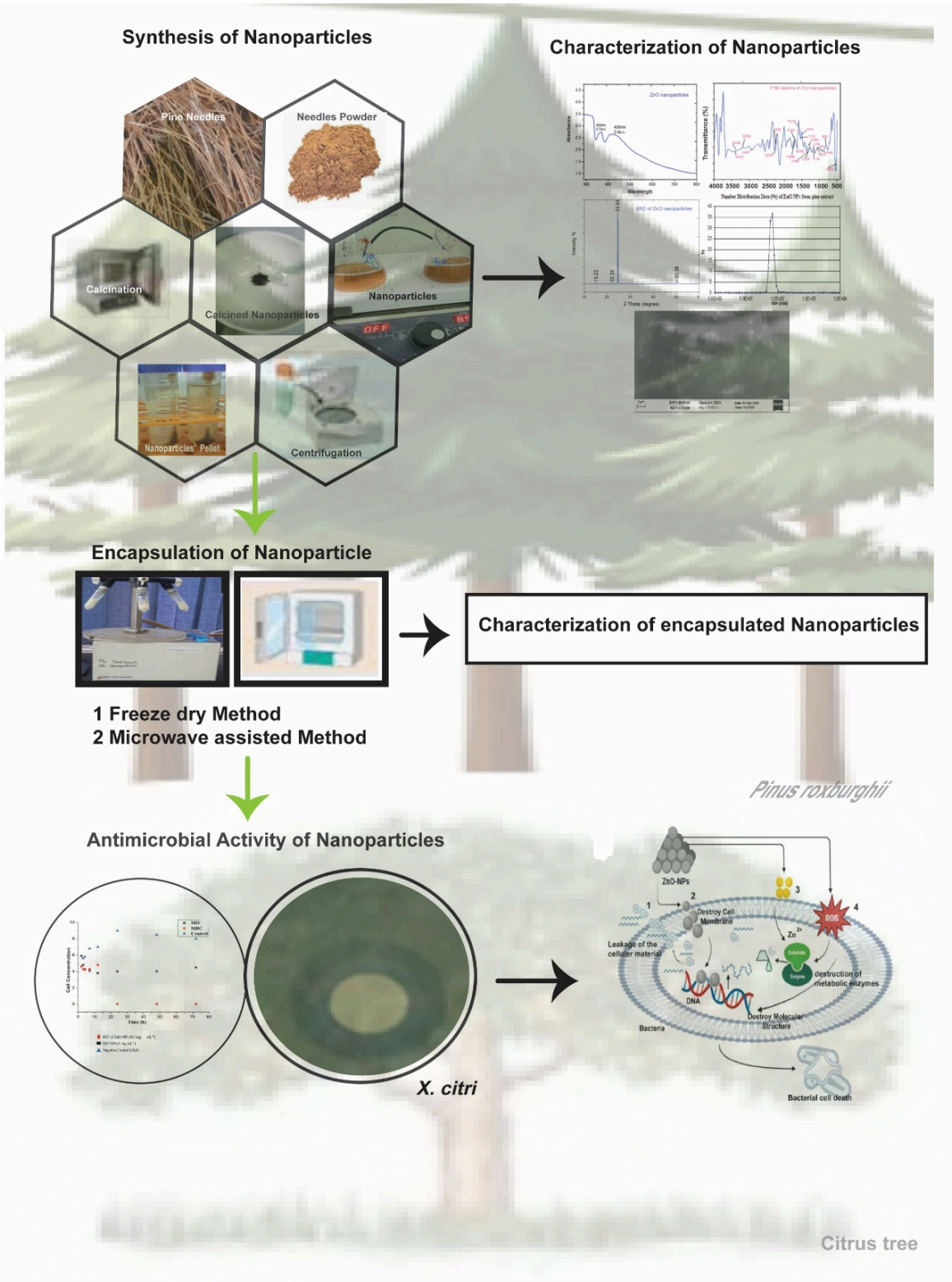

Figure 11

Flow diagram of the research activity conducted during the production of nanoparticles and antimicrobial activity of *Pinus roxburghii*

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